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Telomere elongation in immortal human cells without detectable telomerase activity

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Immortalization of human cells is often associated with reactivation of telomerase, a ribonucleoprotein enzyme that adds TTAGGG repeats onto telomeres and compensates for their shortening. We examined whether telomerase activation is necessary for immortalization. All normal human fibroblasts tested were negative for telomerase activity. Thirteen out of 13 DNA tumor virus-transformed cell cultures were also negative in the pre-crisis (i.e. non-immortalized) stage. Of 35 immortalized cell lines, 20 had telomerase activity as expected, but 15 had no detectable telomerase. The 15 telomerase-negative immortalized cell lines all had very long and heterogeneous telomeres of up to 50 kb. Hybrids between telomerase-negative and telomerase-positive cells senesced. Two senescent hybrids demonstrated telomerase activity, indicating that activation of telomerase is not sufficient for immortalization. Some hybrid clones subsequently recommenced proliferation and became immortalized either with or without telomerase activity. Those without telomerase activity also had very long and heterogeneous telomeres. Taken together, these data suggest that the presence of lengthened or stabilized telomeres is necessary for immortalization, and that this may be achieved either by the reactivation of telomerase or by a novel and as yet unidentified mechanism.

Keywords: cell hybrids/immortal human cells/senescence/telomerase/telomeres

Introduction

Telomeres are the specialized structures found at the ends of eukaryotic chromosomes which protect the chromosome ends against degradation and fusion with other ends. Telomeric DNA is highly conserved throughout evolution and consists of a large number of tandem repeats of short G-rich sequences, TTAGGG in humans and other vertebrates, slime molds and trypanosomes (reviewed in Blackburn, 1991). The telomere hypothesis of cellular senescence, first proposed by Olovnikov (1973), postulates that a progressive shortening of the chromosomes in the somatic cells of multicellular organisms leads to cell cycle exit. As evidence for this, the amount and length of telomeric DNA decreased with continuous passage of normal human fibroblasts *in vitro* (Harley *et al.*, 1990) and were reduced in blood cells relative to sperm (Cooke and Smith, 1986; Allshire *et al.*, 1988; de Lange *et al.*, 1990). Telomere length can also predict the *in vitro* replicative capacity of primary cells; cells with long telomeres had the greatest proliferation potential (Allsopp *et al.*, 1992). Lastly, telomere loss has been associated with ageing *in vivo*: older donors had shorter telomeres than younger donors (Harley *et al.*, 1990; Hastie *et al.*, 1990; Lindsey *et al.*, 1991; Vaziri *et al.*, 1993).

It has been proposed (Harley, 1991) that the immortalization of human cells requires the reactivation of the telomere lengthening enzyme telomerase (Greider and Blackburn, 1985) and that telomerase is normally active in germ cells but not somatic cells (Harley *et al.*, 1992). Simian virus 40 (SV40)-transformed primary human embryonic kidney cells exhibited progressive telomere shortening and lacked telomerase activity up until the time at which the culture entered crisis. An immortal clone arising from this crisis culture had stabilized telomere lengths and telomerase activity (Counter *et al.*, 1992). Similar results have been obtained in human papillomavirus (HPV)-transformed human epithelial cells and human B lymphocytes immortalized by Epstein–Barr virus (Counter *et al.*, 1994a; Klingelhutz *et al.*, 1994). Adenovirus-transformed human embryonic kidney cells also had stable telomeres and telomerase activity following immortalization (Counter *et al.*, 1992). Furthermore, established immortal cell lines do not exhibit telomere shortening, and telomerase activity is present in most immortal human cell lines examined to date (Morin, 1989; Counter *et al.*, 1992, 1994a; Klingelhutz *et al.*, 1994). Telomerase activity was demonstrated in metastatic cells from human ovarian carcinomas (Counter *et al.*, 1994b) and malignant human hematopoietic cells (Nilsson *et al.*, 1994; Counter *et al.*, 1995). The recent development of a sensitive PCR-based assay for telomerase has allowed a larger survey of 100 immortal cell lines and 101 tumors; 98% of cell lines and 90% of tumor biopsies were positive for telomerase (Kim *et al.*, 1994).

In this study, we examined the association between telomerase activation and immortalization. Using the PCR-based telomerase assay, all of 12 SV40-transformed cultures and one HPV-transformed culture were telomerase-negative in the pre-crisis phase. When telomerase activation occurred, it did so only in the rare clones that escaped crisis, in agreement with the three studies on *in vitro* immortalized cell lines cited above. We also used the PCR-based telomerase assay to survey a total of 35 immortal cell lines. Surprisingly, 15 of these cell lines were negative for telomerase. All telomerase-negative cell lines had chromosomes with very long telomeres, suggesting that these cells have been able to overcome telomere shortening through a novel mechanism.

Table I. Telomerase status of *in vitro*-immortalized cell lines

Mechanism of transformation	Cell type	Telomerase –	Telomerase +
SV40	Fibroblasts	WI38-VA13/2RA	BFT-3B[a]
		GM847	BFT-3G
		IIICF-T/A6[a]	BFT-3I[a]
		IIICF-T/B1	BFT-3K[a]
		IIICF-T/B3[a]	GM639
		IIICF-T/C3	GM2096/SV9
		IIICF-T402DE/D2	
	Epithelial cells	BET-3M[a]	BET-3a
			BET-3b
			BET-3K
			BET-1A[a]
			BET-2A[a]
			BES-1A1[a]
			BEAS-2B/R1
			HB56B/5T[a]
	Mesothelial cells	MeT-4A[a]	MeT-5A[a]
HPV	Fibroblasts	IIICF-E6E7/C4	
		IIICF-E6/A1	
		IIICF-E6/A2	
	Epithelial cells		BEPV-3c
	Mesothelial cells		MePV-23F
			MePV-23I[a]
Adenovirus	Epithelial cells		293
CMV	Fibroblasts		CMV-MJ HEL-1
Chemical	Fibroblasts	SUSM-1	
Spontaneous[b]	Fibroblasts	IIICF/c	
		IIICF-.2/A1	

[a]Cultures that were also assayed for telomerase pre-crisis.
[b]Spontaneously immortalized cell lines were derived from a Li–Fraumeni syndrome individual.

Results

Telomerase status of in vitro-immortalized cell lines

A PCR-based assay (designated TRAP, for telomeric repeat amplification protocol; Kim *et al.*, 1994) was used to measure telomerase activity in the 35 human cell lines listed in Table I. This assay detected no telomerase activity in three normal cell strains (data not shown), although it was sensitive enough to detect telomerase activity in the equivalent of 100 HeLa cells (Figure 1), as already reported by Kim *et al.* (1994). Telomerase activity was not detected in 12 SV40-transformed cell strains and one HPV-transformed cell strain in the pre-crisis (i.e. non-immortal) phase of growth (Table I, and examples in Figure 1). Telomerase activity was detected in 20 of the 35 immortal cell lines (Table I).

The absence of telomerase activity did not correlate with the method of immortalization of the cell line. There were examples of telomerase-negative cell lines among those immortalized with SV40, HPV and chemical carcinogens, and in spontaneously immortalized lines. There was no difference in the growth rate of telomerase-positive or -negative cell lines (data not shown). There was also no correlation between telomerase activity and cells of a particular cell type; there were both telomerase-negative and -positive fibroblast, epithelial and mesothelial cell lines (Table I). Cells of the same type and from the same individual may also be either telomerase-negative or -positive (for example, BET-3M, BET-3a, BET-3b and BET-3K are all bronchial epithelial cell lines from the same individual; BET-3M had no detectable telomerase activity whereas the other three lines were telomerase-positive).

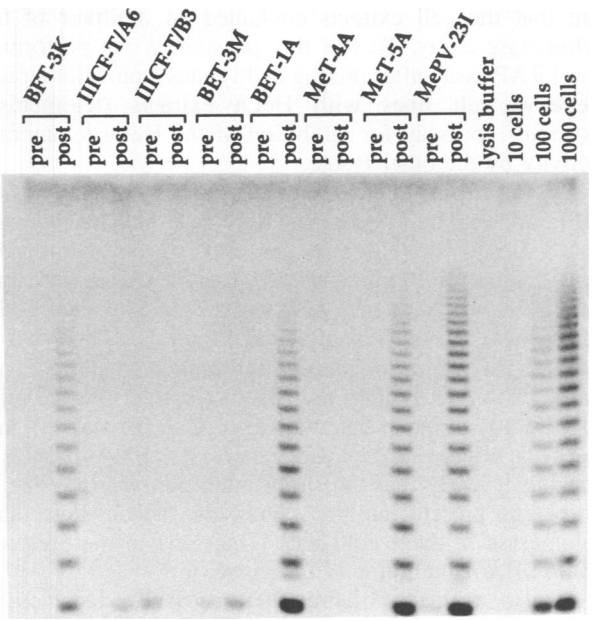

Fig. 1. Telomerase activity in *in vitro*-immortalized cell lines. A PCR-based assay for telomerase activity was used, as described (Kim *et al.*, 1994). Cell lysates were prepared from 10^5 cells using the CHAPS detergent lysis method, and the equivalent of 10^3 cells was used in each assay. The assay uses PCR to amplify the products of telomerase-catalyzed extension of an oligonucleotide primer. Telomerase activity results in a 6 bp ladder when the PCR products are electrophoresed on a 10% polyacrylamide non-denaturing gel. *In vitro*-immortalized cell lines were assayed in the pre-crisis (pre) and post-crisis (post) phases. Extracts representing the equivalent of 10, 100 or 1000 HeLa cells were used as positive controls, and lysis buffer was a negative control with no cell extract.

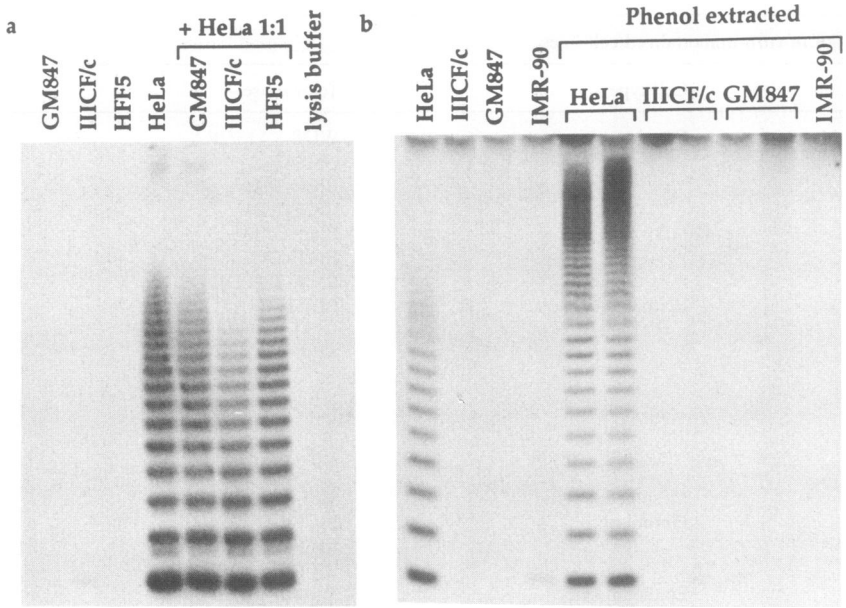

Fig. 2. Telomerase-negative cell extracts do not contain a telomerase inhibitor or an inhibitor of *Taq* polymerase. (**a**) Cell lysates from GM847, IIICF/c or HFF5 (normal human foreskin fibroblast) cells were mixed in a volume ratio of 1:1 with HeLa lysates, allowed to stand at room temperature for 30 min and used in the TRAP assay. Lysis buffer is a negative control with no cell extract. (**b**) The TRAP assay was carried out as described (Kim *et al.*, 1994) with extracts from the cell lines indicated (lanes 1–4). The assay was carried out on the same cell lines, with the telomerase and PCR steps separated by a phenol extraction and ethanol precipitation (lanes 5–11).

Telomerase-negative cell lines do not contain inhibitor(s) of telomerase or Taq polymerase

It seemed possible that cell lines which were apparently telomerase-negative did in fact have telomerase activity, but that the cell extracts contained an inhibitor of the telomerase assay. To test this possibility, we performed the TRAP assay after mixing cell lysates from telomerase-negative cell lines with HeLa extracts (telomerase-positive), to assay for inhibition of the HeLa telomerase activity by the other lysate.

Lysates from all 15 telomerase-negative cell lines or HFF5 (normal human foreskin fibroblast) cells were mixed in a 1:1 ratio with HeLa lysates, allowed to stand at room temperature for 30 min and used in the TRAP assay. None of the telomerase-negative cell extracts abolished HeLa telomerase activity (examples in Figure 2). There was a very slight but reproducible inhibition of activity in all cases. However, boiling the negative extracts before mixing did not abrogate this inhibition (data not shown), and the same effect was seen with lysates of normal fibroblasts, making it likely that the slight inhibition was not due to a specific protein inhibitor. Thus, the lack of detectable telomerase in these cell lines is not due to the presence of an *in vitro* inhibitor of the assay.

It also seemed possible that the lack of detectable telomerase in some cell lines was due to an inhibitor of *Taq* polymerase in the cell extracts, since the TRAP assay is a one-tube reaction. To eliminate this possibility, we separated the telomerase and PCR steps with two phenol extractions and an ethanol precipitation, which should remove *Taq* polymerase inhibitor(s) and allow the products of telomerase to be amplified by PCR. Activity was not restored by this treatment of IMR-90 (normal human fibroblasts) or each of the telomerase-negative cell lines (examples in Figure 2), and the treatment did not diminish telomerase activity in HeLa extracts. The cell line GM847 has also been assayed for telomerase by the conventional telomerase assay, involving extension of a $(TTAGGG)_3$ oligonucleotide by telomerase and direct visualization of the products without amplification by PCR (Morin, 1989), and found to be telomerase-negative in this assay (C.Counter *et al.*, unpublished data), making it unlikely that the absence of detectable telomerase is an artefact of the TRAP assay.

The cell lysates for the TRAP assay are usually prepared in the absence of inhibitors of RNase. Since telomerase is a ribonucleoprotein, we considered the possibility that the telomerase-negative cells had high levels of endogenous RNase, resulting in inactivation of telomerase upon lysis. However, this seems unlikely since inclusion of an RNase inhibitor in the lysis buffer prior to cell lysis did not restore telomerase activity (data not shown).

The TRAP assay was also carried out using a 100-fold range of protein concentration (0.1, 1 and 10 μg total protein per assay) for lysates of all 15 telomerase-negative cell lines and HeLa cells. These concentrations are the equivalent of 200, 2000 and 20 000 cells per assay. No telomerase activity was detected in the telomerase-negative cells at any protein concentration, while the HeLa extract remained telomerase-positive at all protein concentrations (data not shown). Thus the lack of detectable telomerase activity is unlikely to be due to lack of sensitivity of the TRAP assay.

Telomerase-negative immortal cells have very long and heterogeneous telomeres

An analysis of the telomere lengths of selected cell lines is shown in Figure 3. The frequently cutting restriction enzymes *Hin*fI and *Rsa*I digest genomic DNA at points outside, but not within, the TTAGGG repeats present at the ends of human chromosomes. As a result, probing the digested DNA with a $(TTAGGG)_3$ probe detects terminal

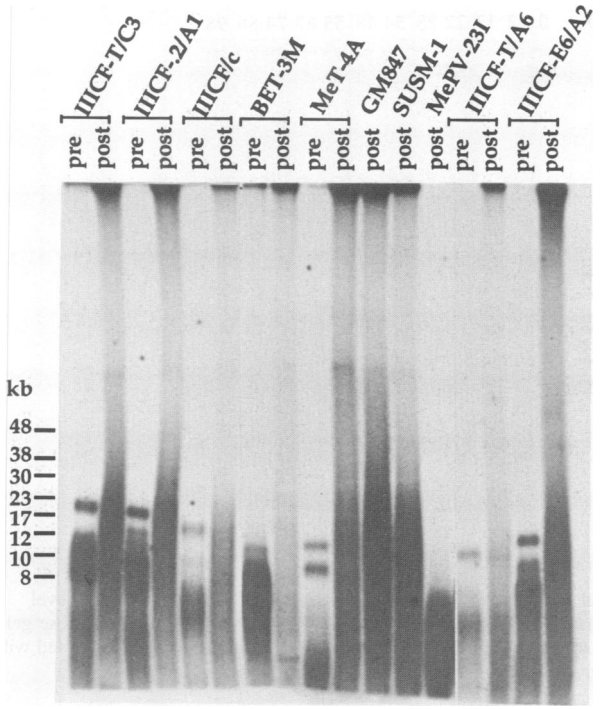

Fig. 3. Pulsed-field gel electrophoresis of TRFs from telomerase-negative cell lines. Genomic DNA samples from the cell lines indicated were digested with *Hin*fI and *Rsa*I and 1 μg electrophoresed on a 1% agarose gel in a pulsed-field electrophoresis apparatus. The gel was dried, denatured, and hybridized to a telomeric probe [γ-^{32}P-$(TTAGGG)_3$]. MePV-23I is a telomerase-positive cell line; all others are telomerase-negative. Cell lines were analyzed in the pre-crisis (pre) and post-crisis (post) phases. Size markers are indicated on the left.

restriction fragments (TRFs) that include all of the terminal repeats and a subtelomeric region (Allshire *et al.*, 1989). The TRFs appear as a smear on a gel (Figures 3, 4 and 5) because both the size of the subtelomeric region and the number of TTAGGG repeats vary on different chromosomes, and because the mean TRF length varies between cells within a population.

The telomerase-positive cell line shown in Figure 3 (MePV-23I) had a mean TRF length of <8 kb. This was comparable with TRF sizes seen by others in immortal cell lines (Counter *et al.*, 1992, 1994a; Klingelhutz *et al.*, 1994). In contrast, the telomerase-negative cell lines all had very heterogeneous TRFs extending over the whole lane, with the peak of the smears from ~10 to 50 kb. All of the telomerase-negative cell lines exhibited very similar TRF patterns, distinct from that of telomerase-positive lines. The very long fragments are not due to incomplete digestion by the restriction enzymes *Rsa*I and *Hin*fI in at least the case of GM847, since a single-locus fingerprinting probe detected two discrete bands, as expected for *Hin*fI digestion (data not shown). The fact that all telomerase-negative cell lines had very large telomeres of about the same size suggests a common mechanism for telomere elongation in these cells.

A comparison of the TRF pattern before and after crisis for seven of the telomerase-negative cell lines is also shown in Figure 3. All of the pre-crisis samples had TRF smears of <12 kb, and bands of 10–12 kb. After crisis, the mean TRF size of each of these cell lines had increased.

The very large TRFs shown in Figure 3 were proven in one case to be telomeric by digestion with the exonuclease *Bal*31, which digests only from the ends of linear DNA molecules. This resulted in a decrease in intensity and size of the TRF smear in DNA from the telomerase-negative cell line SUSM-1 (data not shown). This decrease in size was shown not to be due to non-specific degradation of the DNA by hybridizing the blot with a telomere-unrelated probe, which detected two discrete bands that did not decrease in intensity following *Bal*31 digestion (data not shown).

Telomeric DNA increases after immortalization of IIICF-T/A6 cells

An increase in telomere length in the telomerase-negative spontaneously immortalized IIICF/c cells has been described previously (Rogan *et al.*, 1995). This occurred gradually over many population doublings (PD) *in vitro*; the TRF pattern shown for IIICF/c in Figure 3 was not detected until ~40 PD after immortalization. Similarly, the intensity of the TRF smear in the cell line IIICF-T/A6 gradually increased by 3-fold during the 30 PD following crisis (Figure 4a). This demonstrates that there has been an increase in TTAGGG-hybridizing DNA in the absence of telomerase.

All cell lines established from fibroblasts of the individual IIIC, a Li–Fraumeni syndrome patient, were telomerase-negative and had very long telomeres. Thus, the event which eventually led to greatly lengthened telomeres in the absence of telomerase probably occurred early in the establishment of the IIICF cell lines.

TRF size of GM847 cells remains constant over 100 PD

The telomerase-negative cell line GM847 was passaged *in vitro* and DNA was obtained over the course of 100 PD. TRF analysis of these samples (on a pulsed-field gel) is shown in Figure 4b. Densitometric analysis of the TRF smears shows that the peak of the smears remained constant at 20–23 kb. Thus, over this time course, there was no apparent change in the length of telomeres in a mass population of GM847 cells.

Immortal hybrids from fusions of telomerase-negative and -positive cell lines may be either telomerase-negative or -positive

When immortal cells are fused to different immortal cells the hybrids may remain immortal, but often complementation occurs, resulting in senescence (Pereira-Smith and Smith, 1983). When the telomerase-negative cell line GM847 was fused to the telomerase-positive cell lines BET-1A, HB56B/5T or MeT-5A, most (53/54 total) of the resulting hybrids senesced (Whitaker *et al.*, 1992; Duncan *et al.*, 1993). Some of the senescent hybrids subsequently recommenced proliferation and became immortal (defined as the achievement of >100 PD in culture), consistent with the occurrence of chromosomal segregation in hybrid cells.

In order to determine whether the senescence of the hybrids was associated with a lack of telomerase activity, and whether subsequent immortalization was associated with telomerase reactivation, we determined the telomerase status of the hybrid cells (Table II). There was no definitive relationship between immortalization and telomerase

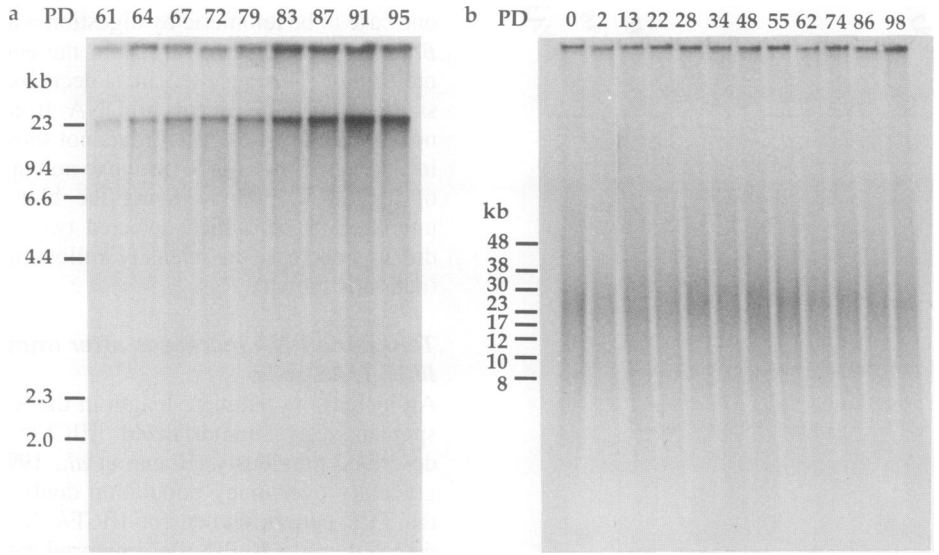

Fig. 4. Analysis of TRFs of the cell lines IIICF-T/A6 and GM847 over sequential passages. **(a)** Genomic DNA from IIICF-T/A6 at the PD level indicated (PD after transfection with SV40 large T antigen) was digested with *Hin*fI and *Rsa*I and subjected to TRF analysis on a 0.8% agarose gel. **(b)** Genomic DNA from GM847 at the PD level indicated (PD levels arbitrarily starting from that at which the cells were thawed) was digested with *Hin*fI and *Rsa*I and subjected to TRF analysis on a 1% agarose pulsed-field electrophoresis gel. Size markers are indicated on the left.

activity. One of six senescent clones displayed telomerase activity. This clone could not be passaged further and hence had not escaped senescence. A senescent hybrid of the cell lines BET-1A and HeLaCOT (both telomerase-positive) was also telomerase-positive (data not shown).

Of the immortal hybrid clones, two were telomerase-positive and three were telomerase-negative. TRF analysis of DNA from each of the immortal hybrids and their parental cell lines is shown in Figure 5. The senescent hybrids could not be included in this analysis due to insufficient cells from which to obtain DNA. The parental line GM847 displayed the TRF pattern characteristic of telomerase-negative cell lines. The three telomerase-positive parental cell lines had mean TRFs from 2.8 to 3.7 kb. The telomerase-negative hybrids (HB56B-5T/GM847 clone Y, MeT5A/GM847 clones E and K) all showed TRF patterns identical to GM847. Of the two telomerase-positive hybrids, one (BET-1A/GM847 clone F) had a TRF pattern intermediate in size between those of its parental lines, and the other (HB56B-5T/GM847 clone L) had a bimodal TRF pattern, with a smear of similar size to each of its parents (Table II).

Table II. Telomerase status of hybrids between GM847 and other immortal cell lines

Cell line/hybrid name	Telomerase activity	Mean TRF (kb)
Parental cell lines		
GM847	–	>20
BET-1A	+	2.8
HB56B-5T	+	3.6
MeT-5A	+	3.7
Immortal hybrids		
BET-1A/GM847 clone F	+	7.6
HB56B-5T/GM847 clone L	+	3.3, >20[a]
HB56B-5T/GM847 clone Y	–	>20
MeT-5A/GM847 clone E	–	>20
MeT-5A/GM847 clone K	–	>20
Senescent hybrids		
HB56B-5T/GM847 clone M	+	ND[b]
HB56B-5T/GM847 clone R	–	ND
HB56B-5T/GM847 clone T	–	ND
HB56B-5T/GM847 clone V	–	ND
MeT-5A/GM847 clone B	–	ND
MeT-5A/GM847 clone N	–	ND

[a]The TRF pattern of clone L is bimodal.
[b]ND: not determined.

Discussion

Recent evidence has been accumulating to suggest that the reactivation of telomerase is necessary to allow somatic human cells to maintain the length of their telomeres and hence become immortal (Counter *et al.*, 1992, 1994a; Klingelhutz *et al.*, 1994). We report here the surprising finding that 15 of 35 *in vitro*-immortalized cell lines were negative for telomerase activity. A recent study of telomerase activity in six *in vitro*-immortalized human cell lines also reported two SV40-immortalized cell lines that were telomerase-negative (Kim *et al.*, 1994). The authors mention that these cell lines had very long telomeres, which is in agreement with our finding that all telomerase-negative cell lines had TRFs of up to ~50 kb. These results suggest that it is indeed necessary for telomeres to be maintained or lengthened if a cell is to become immortal, lending support to the hypothesis that telomere shortening leads to senescence of human cells. Our results indicate that telomere maintenance may be achieved either by the activation of telomerase, or by a novel mechanism. Furthermore, the fact that all telomerase-negative cell lines in this study possessed TRFs of very similar lengths suggests that these cell lines have a common telomere-lengthening mechanism.

The telomere dynamics of a single cell line that lacks telomerase activity have also been described recently (Murnane *et al.*, 1994). Subcloning of this cell line

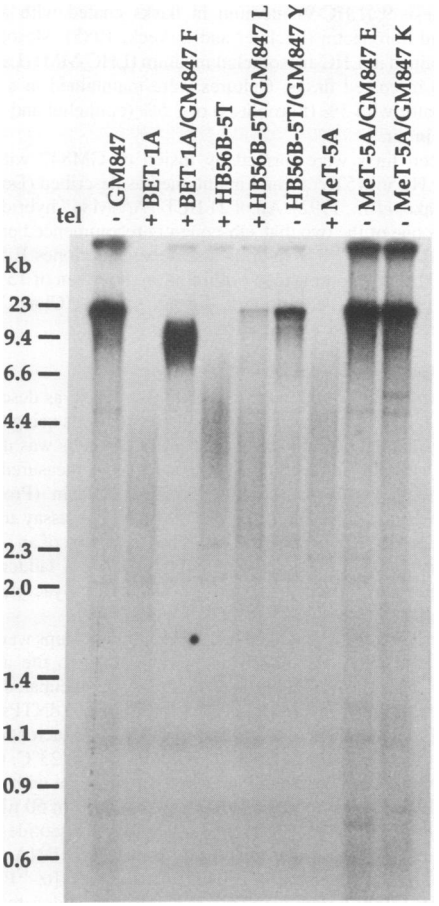

Fig. 5. TRF analysis of immortal hybrid cells and their parental cell lines. Genomic DNA samples from the cell lines indicated were subjected to TRF analysis on a 0.8% agarose gel. The telomerase status (tel) of each hybrid or parent is also indicated. Size markers are indicated on the left.

demonstrated that rapid increases and decreases in telomere length were occurring, resulting in highly heterogenous TRFs resembling those in this study. It is possible that the telomerase-negative cell lines described here had also gained telomere instability, since there was an increase in smaller TRF fragments after crisis as well as larger ones (see Figure 3). If such instability is occurring in individual cells in the cell lines in this study, it is not discernible when TRF analysis is carried out on the mass population of cells (Figure 4).

We considered the possibility that a clone of cells with long and heterogeneous telomeres was present in the pre-crisis population and was selected to survive crisis, rather than telomeres actually lengthening. However, this seems unlikely for two reasons. First, many of the cell lines described were derived from a single transfected colony (including BET-3M, MeT-4A, IIICF-T/A6, /B1, /B3 and /C3, IIICF-T402DE/D2, IIICF-E6E7/C4, IIICF-E6/A1 and /A2). Thus the unusual TRF pattern must have arisen spontaneously at some point during expansion of this colony. Second, there was a progressive increase in TTAGGG-hybridizing DNA after immortalization in the cell lines IIICF/c and IIICF-T/A6 (Rogan *et al.*, 1995, and Figure 4a), and both of these cells lines were clonal at this stage. Therefore, at least in these two cell lines, we can conclude that telomere elongation continues to occur in clonal populations.

It is possible that the apparently telomerase-negative cell lines have telomerase activity that is modulated differently from that in other cell lines. One model is that extensive telomere elongation by telomerase may have occurred, followed by down-regulation of telomerase. The behavior of TRFs in the cell lines IIICF/c and IIICF-T/A6 is inconsistent with this scenario, since they demonstrated gradual lengthening over many PD, rather than a rapid lengthening followed by telomeric shortening. Furthermore, the telomerase-negative cell line GM847 showed a constant TRF smear over 100 PD (see Figure 4b); there should be a 5–10 kb decrease in mean TRF length over this time if the telomeres are decreasing at a normal rate, and this would be clearly visible on the pulsed-field gel. If the same phenomenon is occurring in this cell line as in that of Murnane *et al.* (1994), the constant smear could be masking rapid gains and losses of telomeric DNA in subclones within the population. Nevertheless, these data rule out the possibility that there has been a burst of telomerase activity followed by a gradual decrease in TRF length.

An alternative model is that these cell lines possess telomerase that is active *in vivo*, but mutant in at least one of its subunits. Presumably the mutation has two effects: (i) a dysregulation of telomerase activity *in vivo*, resulting in abnormal lengthening of telomeres; and (ii) the inability of telomerase to use an oligonucleotide as an *in vitro* substrate. This possibility is under investigation.

The very long TRFs observed here may not actually reflect long stretches of telomere repeats, but instead may result from modification of the subtelomeric DNA. A recognized concern with the method used here for assessing telomere length is that the contribution to the TRF length of the subtelomeric fragment is uncertain, and estimates of its size have not been confirmed by sequence analysis of cloned telomere-adjacent regions (Brown *et al.*, 1990; de Lange *et al.*, 1990; Levy *et al.*, 1992). It is possible that some subtelomeric restriction sites were not digested due to DNA modification such as methylation, and the TRF dynamics described here were caused by changes in such modification. However, this is unlikely since the TRF signal intensity increased up to 4-fold in post-crisis cells over pre-crisis (data not shown), which is compatible with at least some of the lengthening being due to the addition of TTAGGG repeats.

The telomerase status of immortal hybrids correlated with their TRF patterns. Immortalization of the hybrids was associated with the presence of either telomerase activity or the alternative mechanism of telomere lengthening (Table II). Telomerase-positive immortal hybrids had TRFs qualitatively similar to other telomerase-positive cell lines, while telomerase-negative hybrids had the very long TRF smears characteristic of telomerase-negative cell lines.

Although we did not detect telomerase activity in normal human cell strains or in non-immortal (pre-crisis) DNA tumor virus-transformed cells, two senescent hybrid clones had telomerase activity. This indicates that maintenance of telomeres is necessary but not sufficient for escape from senescence, at least in the case of hybrid cells.

Other organisms also exhibit telomerase-independent

means of lengthening their telomeres. The yeast *Saccharomyces cerevisiae* has the ability to utilize recombination as a backup mechanism of telomere repair. Non-reciprocal recombination at the boundary between telomeric and non-telomeric DNA resulted in the aquisition of telomeric repeats by very short telomeres in this organism (Wang and Zakian, 1990). The telomeres of *Drosophila* are maintained by transposition of specialized retrotransposons to broken or natural chromosome ends (Biessmann *et al.*, 1990, 1992; Levis *et al.*, 1993). The possibility that one of these mechanisms is occurring in the human cell lines described here is under investigation.

The telomerase-independent mechanism of elongating telomeres may be limited to those cells that become immortal *in vitro*. A survey of 94 tumor-derived cell lines found that all were positive for telomerase (Kim *et al.*, 1994). Similarly, we found that 12/12 tumor-derived cell lines were telomerase-positive (Whitaker *et al.*, 1995). The very long and heterogeneous telomeres in the telomerase-negative cells may be incompatible with cell growth or survival *in vivo* for some reason. Alternatively, mechanisms of immortalizing cells *in vitro* such as SV40 or chemical transformation may play a causative role in initiating this mechanism. In any case, the high frequency of cell lines in this study with no apparent telomerase activity poses many questions for the developing dogma that telomerase activation is required for immortalization of human cells.

Materials and methods

Cells and cell culture conditions

GM847, GM639 and GM2096/SV9 (SV40-immortalized skin fibroblasts) were a gift from Dr O.Pereira-Smith. WI-38 VA13/2RA (SV40-immortalized normal lung fibroblasts) and 293 (adenovirus 5-transformed human embryonic kidney) were obtained from the American Type Culture Collection. CMV-Mj HEL-1 [cytomegalovirus (CMV)-immortalized lung fibroblasts] were a gift from Dr F.Rapp. SUSM-1 are 4-nitroquinoline (4NQO)-immortalized liver fibroblasts, a gift from Dr M.Namba (Namba *et al.*, 1981). BEAS-2B/R1 is a serum-resistant subclone of the cell line BEAS-2B (Ke *et al.*, 1988), which was established following infection of human bronchial epithelial cells with adenovirus 12–SV40 hybrid virus (Reddel *et al.*, 1988). BET-3M, BET-3a, BET-3b, BET-3K, BET-1A, BET-2A and HB56B/5T were established by transfection of human bronchial epithelial cells with the SV40 early region plasmid, pRSV-T (Reddel *et al.*, 1991, 1995; De Silva and Reddel, 1993; De Silva *et al.*, 1994). BES-1A1 is a bronchial epithelial cell line immortalized with SV40 virus (Reddel *et al.*, 1995). BFT-3B, BFT-3G, BFT-3I and BFT-3K are human bronchial fibroblast cell lines immortalized with pRSV-T (De Silva and Reddel, 1993). MeT-4A and MeT-5A are human mesothelial cell lines immortalized with pRSV-T (E.Duncan, unpublished data; Ke *et al.*, 1989). BEPV-3c, MePV-23F and MePV-23I were established by transfection of the plasmid p1321 containing the HPV-16 genes E6 and E7 into bronchial epithelial (BEPV-3c) or mesothelial (MePV-23F, -23I) cells (De Silva *et al.*, 1994). IIICF cells are fibroblasts from an individual with Li–Fraumeni syndrome (Warneford *et al.*, 1992). IIICF-T/A6, IIICF-T/B1, IIICF-T/B3 and IIICF-T/C3 were immortalized with pRSV-T. IIICF-T402DE/D2 was immortalized with a plasmid [pBS-SV(T402DE)] containing the SV40 early region with a point mutation that abrogates p53 binding. IIICF-.2/A1 became immortal after transfection with the control plasmid pRSV.2 (Maclean *et al.*, 1994). IIICF/c cells spontaneously immortalized *in vitro* (Rogan *et al.*, 1995). IIICF-E6E7/C4 was immortalized with plasmid p1321, and IIICF-E6/A1 and IIICF-E6/A2 with plasmid p1436, which encodes HPV-16 E6 but not E7 (K.Maclean, unpublished data).

IIICF cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and gentamicin. All other fibroblasts were maintained in Dulbecco's modified Eagle's (DME) medium with 10% FBS and gentamicin. Epithelial cells were maintained in Laboratory of Human Carcinogenesis-9 (LHC-9) medium in flasks coated with a matrix of collagen and fibronectin (Lechner and LaVeck, 1985). Mesothelial cells were maintained in LHC-mesothelial medium (LHC-MM) (LaVeck *et al.*, 1988), also in coated flasks. Cultures were maintained in a humidified 37°C incubator with 5% (fibroblasts) or 3.5% (epithelial and mesothelial cells) CO_2 in air.

Hybrid cell lines were formed by fusion of GM847 with BET-1A, MeT-5A or HB56B/5T cells and maintained as described (Duncan *et al.*, 1993; Whitaker *et al.*, 1992). All of 21 BET-1A/GM847 hybrids senesced; clone F was one of the two that subsequently recommenced proliferation. All of 18 MeT-5A/GM847 hybrids also senesced; clones E and K were two of the 11 that recommenced proliferation. Fourteen of 15 HB56B5T/GM847 hybrids senesced; clone L did not senesce. Clone Y senesced and then recommenced proliferation.

Telomerase assay

A PCR-based assay for telomerase activity was used, as described (Kim *et al.*, 1994). Cell lysates were prepared from 10^5 cells using the CHAPS detergent lysis method, and the equivalent of 10^3 cells was used in each assay. The protein concentration of lysates was measured using the BioRad Protein Assay kit. Where specified, RNasin (Promega) was added to the lysis buffer (1 U/μl) before lysis. The assay uses PCR to amplify the products of telomerase-catalyzed extension of an oligonucleotide primer. Telomerase activity results in a 6 bp ladder when the PCR products are electrophoresed on a 10% polyacrylamide non-denaturing gel.

For those reactions where the telomerase and PCR steps were separated by phenol extraction, the following modifications to the above assay were made. Two μl of a CHAPS cell extract was incubated initially in the presence of only deoxynucleotide triphosphates (dNTPs) (50 μM), 10× TRAP buffer (Kim *et al.*, 1994) and TS oligonucleotide (0.1 μg) (Kim *et al.*, 1994) in a 50 μl reaction. After 30 min at 23°C, the reaction was extracted twice with phenol/chloroform (1:1), once with chloroform, and ethanol precipitated. The pellet was resuspended in 50 μl containing 10× TRAP buffer, dNTPs (50 μM), TS oligonucleotide (0.05 μg), T4g32 protein (1 μg, Boehringer Mannheim), *Taq* DNA polymerase (2 U, Boehringer Mannheim) and 2 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dTTP (3000 Ci/mmol). This reaction was added to tubes containing CX oligonucleotide under a wax barrier and amplified as described (Kim *et al.*, 1994).

Telomere length analysis

Genomic DNA was extracted from cells using a DNA extraction kit (Stratagene). Twenty μg of DNA was digested with the restriction enzymes *Hin*fI and *Rsa*I (Boehringer Mannheim), extracted once with phenol/chloroform (1:1), ethanol precipitated and resuspended in Tris–EDTA. The digested DNA was quantitated by fluorometry and 1.0 μg was electrophoresed through a 0.8% agarose gel in 1× Tris–borate–EDTA (TBE) buffer at 2 V/cm for 17 h. The gel was dried at 60°C for 2 h, denatured for 30–60 min in 0.5 M NaOH and 1.5 M NaCl and neutralized for 30–60 min in 1 M Tris–HCl pH 8.0 and 1.5 M NaCl. The gel was then hybridized to a [γ-^{32}P]dATP 5′ end-labeled telomeric oligonucleotide probe [γ-^{32}P-$(TTAGGG)_3$]. Hybridization and washing were carried out as described (Counter *et al.*, 1992). The gel was autoradiographed on Kodak XAR-5 X-ray film for 12–24 h at room temperature. Those lanes in which mean TRF was calculated were scanned with a densitometer and the data treated as described (Harley *et al.*, 1990). Mean TRF length was defined as: $\Sigma(OD_i)/\Sigma(OD_i/L_i)$ where OD_i is the densitometer output and L_i is the length of the DNA at position *i*. This method takes into account the greater intensity of signals from larger fragments. The amount of telomeric DNA was calculated by integrating the volume of each smear in ImageQuant software (Molecular Dynamics).

Pulsed-field gel electrophoresis

Genomic DNA samples were digested and quantitated as described above. DNA (1 μg) was loaded on a 1% agarose gel in 0.5× TBE (14×13 cm). The gel was electrophoresed in a CHEF-DR II pulsed-field electrophoresis apparatus (BioRad) in recirculating 0.5× TBE buffer at 14°C. Electrophoresis conditions were: a ramped pulse speed of 1–6 s at 200 V for 16 h. The gel was dried, denatured and hybridized to a telomeric probe as described above.

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